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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Our program objective is to develop simple and rapid methods for detecting, at a cellular level, individual responses to environmental stresses elaborated by exposure to infectious agents such as bacteria and viruses. Our methods are based on transcript profiling and post-translational modification of proteins involved in signal transduction. Our hypothesis is that human cells respond to infectious insults to a genetically predetermined extent by stimulating the expression of sets of genes and activating signaling pathways that provide a specific signature for a given agent. We propose that this response will determine the outcome of the infection. We will test this hypothesis by developing custom cDNA and protein arrays designed to detect cellular responses to infectious agents. These will be tested using RNA and protein isolated from tissues sources most likely to be exposed. Our methods will allow development of rapid quantitative detection devices to measure exposure and response to biological warfare, bioterrorism or emerging agents enabling appropriate triaging and medical intervention to save lives and to avoid unnecessary treatments.

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Introduction

There been recent recognition of an increased risk posed by the use of weapons of biological warfare and bioterrorism. By enhancing our ability to respond by rapidly identifying exposed populations and through the triaging of high risk individuals we can overcome both deliberate and naturally occurring pathogen releases. Restricting distribution to exposed and susceptible individuals can also eleviate the shortfall in therapeutic products (when these exist). The rapid testing methods we propose here have as their longterm objective, the detection and identification of individuals who have been exposed to any one of a number of biological agents and at the most risk for succumbing from this exposure. We hypothesize that specifc gene and protein expression exist for viruses and bacteria. To test this we will establish the gene expression and protein signatures of different important viruses and bacteria that serve as surrogates for highly pathogenic agents. For viruses in which cell culture models exist, RNA will be isolated from virus and bacteria-infected cells. In addition, we will obtain RNA from infected primary human cells and mouse models. RNA isolated from mammalian cells will be converted to dye-labeled complementary DNA or RNA and the labeled nucleic acids will be used to probe DNA microarrays representing thousands of human genes. Viral and bacterial specific signature profiles will be thus obtained. We will construct a composite virus and bacteria induced gene chip as a component of this project. The proposed work will result in technology capable of determining if individuals have been exposed to a life-threatening virus or bacteria and will identify the particular infectious agent by virtue of the signature transcripts induced in the cells. Signal transduction pathways activated by bacterial infection of human cells will also be identified and single chain antibodies against a specific signaling component generated.

Body (progress report)

Technical Objectives

Assembly and testing of prototype sentinel cDNA and protein microarrays for detecting signatures of biological agents

A Generating viral specific gene expression profiles from select groups of virological and bacterial agents involved in bioterrorism

Because of restrictions placed on access to and transport of select groups of agents we had to modify our original proposal to use only surrogate agents at our institution (this was done in consultation with the DOD). Two viruses were chosen, vaccinia, as a surrogate for small pox and influenza, because of its potential both as a bioterrorism weapon and because of its public health importance. Both these agents can be used in our BSL approved laboratories and both can be modeled in mice. Importantly, vaccination programs with vaccinia have been implemented recently offering the possibility of obtained specimens from vaccinated individuals to test our arrays and expand the numbers of genes identified as potential sentinels. Experiments with Ebola have been conducted in collaboration with Dr Jason Paragas USAMRIID laboratory in Ft. Detrick, Maryland.

1) *in vivo* profiling of response to vaccinia infection.

C57 BL6 mice were infected intranasally with vaccinia virus and brain RNA was extracted at 3 days post-infection. Expression levels of over 15 thousand genes were measured relative to

levels in brain RNA from uninfected control animals, using the National Institute of Aging 15K mouse cDNA microarray (Tanaka *et.al.,* 2000).

In a typical experiment a total of 92 genes exhibited a 3 fold or greater level of induction in infected brain whilst 9 genes were repressed 2 or more fold. These data are summarized in Table 1. Included in the upregulated class are Hsp86 and 15 mitochondrial genes. Only 35 genes were induced to a level above 5. In addition to Hsp86, this set of highly upregulated genes encodes a number of proteins not previously linked with disease, such as the carbonic anhydrase related polypeptide (>30 fold induced) and erythroid differentiation regulator (12 fold induced). Whether these genes could represent specific markers of vaccinia infection will be further tested. A Northern blot confirmed high level induction of erythroid differentiation regulator mRNA. A more definitive set of experiments is now underway to validate and extend these initial findings. This will incorporate multiple time points post-infection and 5 replicate treated and control animals at each time point. In addition to brain, other potential target organs including lung and spleen will be examined.

Table 1 Genes differentally expressed in vaccinia virus infected mouse brain

Gene	Fold Induction	Accession Number
carbonic anhydrase-related polypeptide	45.8	BG072807
erythroid differentiation regulator	15.5	BG066172
adaptor protein Lnk	13.5	BG078449
calmodulin dependent phosphatase catalytic subunit (Cam-Prp)	8.9	P48453
heat shock protein, 86 kDa 1 (Hsp86-1)	6.7	BG063605

Gene	Fold repression	Accession Number
KIAA1606 protein	13.5	BG084977
peter pan (Drosophila) homolog	5.2	BG086038
A10 mRNA	3.8	BG064679
peroxisomal integral membrane protein PMP34	3.6	BG078796
Mitochondrial ATPase subunit 6	3.4	BG072772

2) *in vitro* **profiling of response to influenza virus infection in cultured human airway epithelial cells (HAEC).**

HAEC infected with influenza virus were harvested at 1, 8, 24 and 72 hrs post infection and RNA extracted. Gene expression levels were determined relative to RNA from uninfected control cells with an in-house generated microarray comprising approximately 1000 known interferon stimulated genes (ISGs), 1500 genes containing adenylate-uridylate-rich elements (AREs) and 300 genes responsive to treatment with the viral analogue dsRNA.

Table 2 Genes differentially expressed in influenza infected HAEC

Time post-infection (hrs)	Genes min 2 fold upregulated	Genes min 2 fold downregulated
1	115	94
8	76	16
24	66	56
72	4	15

Distinct waves of differential gene expression were observed at 1, 8 and 24 hours post-infection each involving largely non-overlapping sets of genes. The numbers of up and down-regulated genes are summarised in Table 2. Notably, both activation and repression of large numbers of genes in influenza infected Hela cells (Geiss et.al., 2001) and a transformed lung epithelial cell line (Geiss et.al., 2002) have been previously reported.

3) Determining the gene expression profile of Ebola virus infected cells.

We performed preliminary experiments that are aimed at determining gene expression profiles of several different species of Ebola virus in human cells. For these experiments we are using derivatives of human HT1080 fibrosarcoma cells that are either normal or mutated for Jak1 kinase required for an interferon antiviral response. Cell lines were characterized for their IFN response by performing gene array experiments. In addition, the same cell lines were sent to USAMRIID laboratory in Ft. Detrick, Maryland for Ebola virus experiments (performed by Dr. Jason Paragas). Reston, Sudan and Zaire species of Ebola virus were analyzed.

Table 3. Cell lines used for Ebola virus experiments.

HT1080 Derivative:	Jak1 Status	Susceptibility to Ebola Virus
2C4 (wild type clone)	positive	Normal
U4A (JAK1 negative)	negative	Reduced

U4A:JAK1M12H:T889> D clone 3	missense substitution mutation	Reduced
2C4 JAK1K>E clone C	kinase dead Jak1	Reduced
2fJAK1deltaB clone 5	truncated Jak1	Enhanced
U4A:JAK1M12H(wild type) clone3	Jak1 reconstituted	Reduced

Table 4. Replication of different species of Ebola virus in different HT1080 derived cell lines.

2C4 (wild type clone)	Virus	MOI 1	MOI 0.001
	Reston	1.7×10^4	0
	Sudan	1.4×10^7	1.0×10^3
	Zaire	$>10^7$	$>10^7$

U4A (JAK1 negative)	Virus	MOI 1	MOI 0.001
	Reston	0	0
	Sudan	1.4×10^4	0
	Zaire	1.6×10^7	8×10^3

U4A:JAK1M12H:T889>D clone 3	Virus	MOI 1	MOI 0.001
	Reston	0	0
	Sudan	30	0
	Zaire	6×10^5	1.9×10^2

2C4 JAK1K>E clone C	Virus	MOI 1	MOI 0.001
	Reston	0	0
	Sudan	7×10^3	0
	Zaire	$>10^7$	$>10^7$

2fJAK1deltaB clone 5	Virus	MOI 1	MOI 0.001
	Reston	$>10^7$	ND
	Sudan	1.5×10^6	1.5×10^4
	Zaire	1.7×10^8	1.5×10^5

U4A:JAK1M12H (wild type) clone3	Virus	MOI 1	MOI 0.001
	Reston	0	0

	Sudan	0	0
	Zaire	1.9×10^5	1.0×10^3

c.   **Significance**

Results show that the cells harboring the truncated Jak1ΔB, have enhanced ability to replicate the Reston and Zaire species of Ebola virus. Four cell lines have a reduced ability to replicate Ebola virus. By comparing gene expression profiles of these cells, we will identify genes that are induced in susceptible and resistant cells. We will correlate gene expression patterns with susceptibility to the antiviral effect of interferon in the different cell types. We also hope to obtain Ebola virus species specific differences in the gene expression profiles.

B-G Assembly of sequence-verified cDNA arrays, manufacturing, probe preparation, hybridization, scanning and data analysis

We have successfully replicated the National Institute of Aging 15K mouse cDNA microarray (Tanaka *et.al.,* 2000) and used this for the experiments in mice described above. We have also constructed a microarray comprising approximately 850 known human interferon stimulated genes (ISGs), 1500 human genes containing adenylate-uridylate-rich elements (AREs) and 300 human genes responsive to treatment with the viral analogue dsRNA and used these for experiments with influenza virus infected cultured human airway epithelial cells.

H-I Preparation of synthetic peptides that are the activated forms of key signaling molecules Construction and arraying of single chain antibodies to cell signaling components.

The overall objective of this task is to assemble and test prototype sentinel protein microarrays for detecting signatures of biological agents. During this first year of study, different cell types infected with a select group of virological and bacterial agents that could act as surrogates for agents involved in bioterrorism were assembled and protein extracted from control and infected cultures. To identify the molecular signaling pathways activated by bacterial and viral pathogens and their products different relevant cell types (lung epithelial, intestinal epithelial, oral eiptheial cells and macrophages) were used. The major stress-activated pathways in lung epithelial cells and in intestinal epithelial cells were identified. The lung trophic viruses Respiratory syncitial virus (RSV) and human parainfluenza virus (HPIV-3) and the bacterial pathogen Salmonella typhimurium and the bacterial protein flagellin were used to stimulate responses in tissue culture cells. As a by-product of these studies, it was found that flagellin is the main proinflammatory mediator in both intestinal epithelial cells and also in lung epithelial cells. Additionally and perhaps more importantly we have uncovered a novel anti-viral innate host response that requires the activation of the NF-kB transcription factor and expression of one of its unknown target genes. This unknown gene(s) can turn a mildly cytopathic virus (HPIV-3) into an extremely virulent one similar to RSV. Also, triggered expression of this NF-kB target gene by a number of means prior to RSV, or influenza A severely limits the

infectiveness of these and other negative strand RNA viruses. These findings are significant in that they point the way that a poorly cytopathic virus can be turned into a virulent one without genetic manipulation. As importantly, these results also point to the existence of a factor(s) that can negate the effects of virulent negative strand RNA viruses and could have broad-spectrum protective effects among a number of current Category A biologicals. We are also working on further delineation of additional activated signaling pathways in lung and intestinal epithelial cells (JAK-STAT, growth factor, ER stress) comparing responses in these cells in with those to viral and bacterial challenges. One problem encountered is the ability to compare results of one immunoblot with others and get an idea of the relative strength of how efficiently a particular signaling pathway has been activated. This problem arises from loading multiple samples on multiple gels and performing multiple protein transfers. A solution to this problem may exist by using a new protein transfer technique developed by scientist at the NIH that has recently been commercialized (Kodak BioMax Multi-blot kit). This technique allows the proteins from one gel to be transferred to as many as ten membranes so that protein loading is identical between membranes. Currently we are evaluating this system for its utility in reproducibility and will allow for conservation of samples of limited protein abundance (some virally infected cells, i.e., influenza A) and initial results appear encouraging.

The development of single chain antibodies directed against phosphorylated and non-phosphorylated peptides of key signaling molecules that we have previously identified also proceeded. Originally, our protocol called for making single chain antibody libraries from immunized animals and then isolating the specific scFvs from those libraries using screening procedures utilizing the specific phosphorylated or non-phosphorylated peptide. Initial attempts to isolate a phospho-specific scFv antibody from a scFv library prepared from a previously immunized animal failed to produce a phospho-specific scFv while a scFv to the non-phosphorylated peptide could be generated. We believe that this will be the case for a number of the phospho targets. The problem most likely lies in the fact that the complexity of our home-made libraries is at the most slightly greater than 10^8 members. To circumvent this problem we have contacted and have acquired a large human scFv library (over 10^{11} members) from Cambridge Antibody Technologies (CaT) (Cambridgeshire, UK). CaT is one of the leading phage display, single chain antibody biotechnology companies in the world. CaT has created libraries of antibody genes collected from the blood of healthy individuals. These libraries contain the genes for over 100 billion distinct antibodies. CaT has used these gene libraries as the basis for the production of phage antibodies, created by phage display. The larger the antibody libraries, the more chance there is of them containing high quality antibodies that will bind to any given target molecule. By mixing the genes that code for the variable parts of an antibody (V genes) to give further combinations and inserting them into phage, CaT has created phage antibody libraries that now contain over 100 billion distinct phage antibodies. These libraries are in vitro immune system equivalent in size to the immune systems of 10 human beings. The size of CAT's libraries allows isolation of antibodies to potential targets rapidly and efficiently. Currently, we have used the large BMV CaT scFv library to screen a number of the non-phospho and phospho peptides. We have been able to generate positive binding scFvs for phospho- and non-phospho peptide pairs so far tested and are in the process of identifying the best binders of this group. We will use ribosome display to isolate any scFvs against phospho-

peptides that fail to be isolated using the other CaT scFv library and will utilize ribosome display to strengthen the binding characteristics of any weakly binding phospho-specific scFv's generated.

The key cell signaling proteins initially targeted for ribosome display are the I kappaB kinase (IKK), the mitogen-induced kinase p38, a signal transducer and activator of transcription (STAT3), and the dsRNA activated kinase PKR. Together these proteins play pivotal roles in cell development and differentiation as well as in maintaining cell homeostasis. Inappropriate activation of these proteins is associated with exposure to infectious agents. All four proteins exist in an inactive state that can subsequently become activated by phosphorylation at well-defined serine, threonine and/or tyrosine residues in each protein. The goal is to generate antibodies that specifically recognize these essential residues in their phosphorylated state and so will detect the activated full-length proteins. Towards this end, peptides have been synthesized with the pertinent phosphorylated amino acids to be used as antigens against naive immune libraries (Figure 1).

We have acquired access to three naive immune library (coded CS, BMV, and DP47) from collaborators at CaT. These libraries consist of single-chain fragments of the variable region of antibodies amplified as PCR products. The libraries have been formulated so that they can be used with ribosomal display technology. For this protocol the DNA libraries have been engineered to remove the translation stop codons then fused to a linker that permits the translated proteins to fold outside of the ribosome complex while retaining the linker in the ribosome tunnel. The translated products from such constructs form a complex consisting of the ribosome, mRNA and translated protein. This ternary complex is used directly for binding selection against target antigens. Binders are subsequently recovered by dissociating the complex, purifying the mRNA and performing a reverse transcriptase reaction followed by PCR. Through cycles of transcription, translation, antigen-affinity selection, and PCR antibodies can be rapidly selected without antigen purification.

The resolving power of this *in vitro* technology is defined by the diversity of the binding library as, unlike cell-based display techniques such as yeast two hybrid or bacterial display which are constrained by transfection efficiencies, ribosomal display is limited only on the scale of the *in vitro* translation used. The immune libraries obtained from CaT express 10^{14} members, making them the most diverse libraries available.

The ribosomal display libraries have been tested in preliminary experiments to generate enriched binders for phosphorylated peptide antigens. Biotinylated phosphorylated peptide antigens were used (at 100nM) in a solution selection then recovered with streptavidin beads. After this first round of selection excellent distinctions were observed for the IKK peptide compared to controls without antigen from all libraries (figure 2a). Clear distinctions were also apparent with the p38 peptide and the CS and BMV libraries (figure 2b). Early selections with the STAT3 peptide showed no apparent increased binders over those of controls without antigen. However, subsequent reselection of outputs from the first round with reduced (10nm) concentrations of the STAT3 peptide do show enrichment (figure 2c).

Continuing selection rounds are required to sequentially remove non-specific binders, such as antibodies that bind non-phosphorylated or non-specific phosphorylated residues, from

the enriched pool. Initial outputs from selections with the phosphorylated IKK peptide maintain good enrichment for this antigen (used at 10nM) after competitive removal of binders to the respective non-phosphorylated peptide (used at 100nM) (figure 3).

Figure 1: Amino acid sequence of peptide antigens. Each antigen was synthesised as a phosphorylated (phosphorylated residues are underlined) and non-phosphorylated peptide, both with and without a biotin tag.

IKK   **KELDQGSLCTSFVGTLQY-COOH**

STAT3   **NTIDLPMSPRTLDSL-COOH**

P38   **RHTDDEMTGYVATRWYR-COOH**

PKR   **SLKNDGKRTRSKGTLRYMS-COOH**

Figure 2: Outputs from ribosomal display showing enriched binders from three naive immune libraries (CS, BMV, DP47) to three biotinylated-phosphorylated peptides from the proteins IKK, p38, and STAT3.
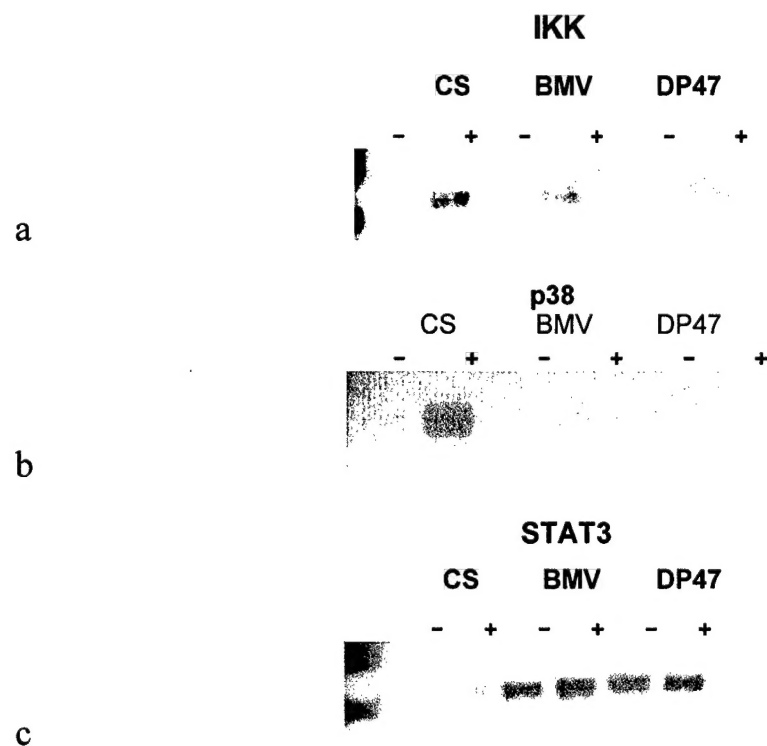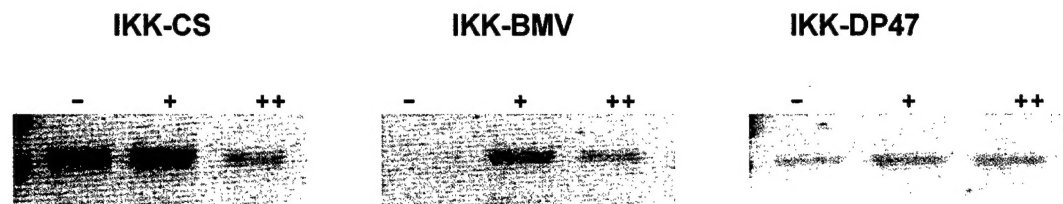
Figure 3: Outputs from a second round of selection showing binders to the phosphorylated IKK peptide isolated previously then reselected against the biotinylated-phosphorylated IKK peptide alone at 10nM (+) and this peptide with the respective non-biotinylated non-phosphorylated peptide used at 100nM (++) as a competitor.

IKK-CS **IKK-BMV** **IKK-DP47**

J   *Molecular signaling pathway characterization of model stimuli*

These experiments require the production of fully characterized single chain antibodies and good progress has been achieved as outlined above.

K   *Feasibility of large scale screening of airway epithelium exposed to biological agents*

In general, exposures to biological agents in bioterrorism in humans occur first by the aerosol route with inhalation into the respiratory system. However, the feasibility of using these types of samples for large-scale detection of exposures is unknown. In this context, this task's objectives include:

> (1) Provision of sterile lung epithelial cells for evaluation of feasibility of use of primary cells in experiments.
> (2) Expansion and maintenance of primary lung cell cultures for use in *in vitro* studies of toxin or infectious agent exposure.

Specifically, this task allows access to human clinical samples allowing the direct study of the effects of toxins on primary human cells and tissues. We are using a lung sample repository which has been maintained in the Lung Biology laboratory over the previous 8 years. Nontrackable sample numbers, which are not linked to the donor, are used as unique nontraceable identifiers. This task has been successful during this interim period in establishing the lung epithelial cells in culture, confirming epithelial phenotype, lack of infectious contaminants, and providing epithelial cell RNA in sufficient quantity for microarray experiments using *in vitro* exposure of primary cells to Influenza.

For evaluation of airway biomarkers, initially cells were grown to approximately 85-90% confluence in 100 mm dishes and infected with Influenza A2 Japan virus using 10 viral particles/cell for an estimated 8×10^6 cells/plate. Infection of cells was performed using sterile techniques in the BSL2 virus facility in the tissue culture hood. RNA was prepared using phenol/chloroform purification and extraction methods. RNA concentrations and quantities were determined for each of the samples, which were then confirmed on agarose gels.

Three experiments have been completed. Total RNA extracted from plates was 11 ± 4 ug/1×10^6 cells. RNA was of good quality with no degradation as evaluated by gel electorphoresis.

L   Human Clinical Samples

The Cleveland Clinic Lung Biology Program has an established active research program with lung cells and tissue acquired and cultured over the previous 8 years. Human airway epithelial cells (HAEC) are grown on collagen-coated dishes, in specialized serum-free media (Clonetics). We have shown that cell cultures of HAEC lead to pure epithelial monolayer cultures. The epithelial nature of cultured HAEC was confirmed by morphology and positive reactions to anti-cytokeratins, which are epithelial cell specific. We characterize cells in culture as epithelial using anti-cytokeratin stains [(AE1/3 (Biogenex), 34BE12 (Dako)] while we have excluded contamination by mesenchymal cells, macrophage, lymphocyte cells by absence of staining for vimentin (Neomarkers), CD68 and CD3 (Dako). Non-transformed HAEC are limited to ±25 population doublings, a culture span of about 1-month and a maximum of 4 to 5 passages. All cells were mycoplasma tested and confirmed negative before use in experiments.

Key research accomplishments

We have successfully constructed cDNA arrays representing mouse and human genes. The mouse set contains 15,000 cDNA and has been used to monitor the induction of gene expression in vaccinia virus (a small pox surrogate) infected mouse brains over the course of several days. Novel upregulated genes were identified and one (erythroid differentiation regulator confirmed by northern blotting. An array (2,800 cDNA) was also constructed for analyzing infection-induced genes in human cells and used to profile influenza virus infection in cultured primary human airway epithelial cells (HAEC). Expression profiles were compared to those previously reported for influenza virus infected cells and unique genes identified. A mechanism was identified by which a poorly cytopathic virus can exhibit enhanced virulence without genetic manipulation. Ribosomal display technology was used in preliminary screens to raise antibodies against different key cell signaling proteins including STAT3 and IKK.

Reportable outcomes

No manuscripts submitted to date.

Conclusions

We have successfully adapted sophisticated technology and used this to begin to address the major objectives of our proposal, i.e. the description of genes that could be developed as sentinel markers of infection with specific agents. We have also shown that single chain antibodies can be derived against key cell signaling targets using ribosomal display. Planned work includes further analysis of gene expression profiles in influenza and vaccinia virus infected mouse and human cells and the analysis of ebola virus infected patterns of expression in different mouse organs.

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Appendices

None